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PURE CULTURE OF WILD TOUGH MUSHROOM COLLECTED FROM TAY NINH PROVINCE OF VIET NAM

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ABSTRACT

Pure cultures of wild edible mushroom have drawn much attention all over the world for exploitation and conservation of precious genetic resources. In this study, a wild strain of tough mushroom, which has been used as human food in Tay Ninh Province, was collected. The strain was purely cultured on agar medium and grown on different culture media for mycelial growth and fruiting body production. It was also classified based on morphological characteristics and molecular methods. The results show that this wild tough mushroom belongs to *Lentinus squarrosulus* (Polyporaceae). The best mycelial growth rate (8.3 mm/day) was recorded on the unhusked rice medium containing $CaCO_3$ (1 %) and $MgSO_4$ (1 %). In both of rubber tree sawdust medium supplemented with corn bran (10 %) and the one supplemented with earthworm fertilizer (5 %), the mycelial growth rates were significantly similar (8.4 mm/day) and achieved full colonization of the both supplemented sawdust bags (each 1 kg) after 24 days of incubation. The fresh fruiting bodies yielded 398 - 412 g/bag of the both sawdust media.

Keywords: Lentinus squarrosulus, pure culture, tough mushroom, wild edible mushroom.

1. INTRODUCTION

Wild edible mushrooms have been collected across the globe for thousands of years as their medicinal and nutritional values. Most mushrooms are known to be rich in proteins, fibers, vitamins, minerals, trace elements and low/no calories and cholesterol [1]. Out of 15000 known species of mushroom in the world, 2000 species are considered safe for human consumption and approximately 650-700 are known to possess medicinal properties [2, 3]. However, many wild mushroom species have been on the brink of extinction due to agriculture and forestry practices every year. Therefore, collection and identification of mushrooms in the wild is important for the studies of mushroom diversity and their role the environment. The activities would also provide the genetic variability required for breeding new strains with higher yield and/or phenotypic traits useful for mushroom industry and research purposes [4, 5].

In Viet Nam, natural resources of mushrooms are evaluated to be very diverse. Many new mushroom species have been found and identified in Viet Nam and some of them have been classified as new species in the world [6-9]. Annual rate of deforestation and environmental pollution are always major threats to biological systems and the diversity of wild species. However, there has not been adequate attention given to studies on collection and identification of wild edible and medicinal mushrooms. In addition, many mushroom species for food and/or medicinal purposes are foreign imported and widely available leading to less attention to domestic wild species. Therefore, in the present study, a wild edible mushroom was collected from Tay Ninh, a south eastern Province of Viet Nam. The strain was pure isolated and identified. Culture conditions for mycelial growth and basidiocarp production of the isolated strain was also investigated and reported to aim to genetically conserve and introduce native wild mushrooms to our mushroom industry.

2. MATERIALS AND METHODS

2.1. Materials

Fruiting bodies of the wild tough mushrooms were collected from decaying woods at different areas in Tan Bien, a rural district of Tay Ninh Province in the southeast of Viet Nam. The internal tissues of the fruiting bodies were cultured on PGA (potato glucose agar) plates to obtain and maintain the pure mycelia. For DNA extraction, the mycelia were subcultured to 50 mL PGB (potato glucose broth) and incubated for 7 days at 25 ^{o}C with a shaking at 100 rpm.

2.2. Methods

2.2.1. Classification method

Shape, size, color, and structure of pileus, gill and spore of the mushroom were observed and determined according to taxonomy keys described by Abdullah [10], Le Ba Dung [11], Senthilarasu [12] and Trinh Tam Kiet [13], to classify the mushroom.

Genomic DNA was extracted from the pure mycelial preserved in CTAB (cetyltrimethylammonium bromide) based on the method of Henrion et al. [14]. The ITS (internal transcribed spacer) region of rDNA of the strain was amplified with 2 primers for PCR reactions, ITS1-F (5′CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4-R (5′TCCTCCGCTTATTGATATGC-3′) [15, 16]. The PCR reaction was performed in 25 µl total volume, containing 1µl genomic DNA, 1µl of each primer, 12.5µl master Mix 2X (0.4 mM of dNTPs, 10X PCR buffer with 3 mM $MgCl_2$, and 0.1 U/µl Taq polymerase). The thermocycling conditions consisted of an initial denaturation at 94 ºC for 5 minutes, followed by 35 amplification cycles at 94 ºC for 45 seconds, 58 ºC for 50 seconds and 72 ºC for 1 minutes, and a final extension at 72 ºC for 10 minutes. The amplified product was purified from agarose gel 1 % using QIA quick PCR purification Kit and sequenced at Nam Khoa Biotek Company. The resulted gene sequence was subjected to NCBI BLAST to identify the closest related sequences.

2.2.2. Effects of different grains on spawn production

The three cereal grains, unhusked rice, brown rice and corn were used as substrates for the preparation of grain spawns. After washed and soaked in water for overnight, the grains were boiled for 15 minutes, subsequently air dried and supplemented with $CaCO_3$ (1 %) and $MgSO_4$

(1 %) or without supplementation. These grains were filled into polypropylene bags (7 x 24 cm). After sterilized for 15 minutes at 121 °C in 1 atm, the substrate filled bags were inoculated with five uniform sized mycelial discs (5 - 6 mg of mycelia/disc) and incubated at 30 ± 2 °C. The mycelial growth in the different bags of the substrate materials was compared and recorded in four replicates.

2.2.3. Effects of substrates on the growth and yield of fruiting bodies

Three different locally available materials such as rice straw, bagasse and rubber sawdust were selected as substrates for fruiting body production. Rice straw and bagasse were soaked in a tank containing lime water (0.35 %) for 10 minutes. The piles of the materials were incubated for 7 days and turned for aeration three times a week. The piles of rubber tree sawdust were mixed with lime water 1 % and incubated for 24 hrs. Finally, these all piles were mixed with $MgSO_4$ (0.3 %) and KH_2PO_4 (0.2 %). These substrates were packed into polyethylene bags and pressed to form a cylindrical cake. The substrate bags were sterilization at 100 °C for 12 hrs. The spawn bags were incubated at 30 ± 2 °C. Sixty bags of each substrate were planted in three replicates. Fruiting body yields in the different bags was compared and recorded.

2.2.4. Nutritional analysis

The proximate compositions of the mushroom fruiting bodies were examined. The ash content was obtained from 10 g sample in the muffle furnace after 4 - 6 hours at 600°C. N total was determined by the Kjedahl method. The crude protein was obtained using the conversion factor (total N x 3.48) and the Soxhlet device to extract the crude fat and carbohydrate determined by the phenol sulphuric acid method of Dubois et al. [17].

2.2.5. Statistical analysis

All experiments conducted were repeated in three replicates. The means ± SD of Mycelial growths and fruiting body yields were compared using ANOVA and the Bonferroni–Dunn's test for multiple comparisons (SAS 9.3, 2002–2010; SAS Institute Inc., Cary, NC, USA).

3. RESULTS AND DISCUSSION

3.1. Morphological features and molecular characterization of the wild tough mushroom

In the wild, tough mushroom is a white rot fungus which thrives saprophytically on dead wood. The mushroom was found to grow singly or more commonly in groups or clusters from April to November in the forest at Tan Bien District of Tay Ninh Province (Figure 1A). The fruiting body has a thin, white and funnel-shaped pileus (2 – 5 cm in diameter). The pileus surface was covered by scales having recurved fibrillose to floccose fibrillose. Gills (or lamellae) are deeply decurrent, unequal, tough and white. The gill edges are smooth. The stipe is central or eccentric (2 – 5 cm long and 0.5 – 1.0 cm broad), concolorous with the pileus, fleshy, tough and cylindrical to slightly tapering downwards and solid. The stipe surface is covered by floccose fibrils over the entire surface (Figures 1A & 1C). Context white consisting of a dimitic hyphal system. Generative hyphae have thin-walled with clamp-connections. Skeleto-ligative hyphae have thick-walled and hyaline. Basidia bear 4 sterigmata, claviform and hyaline. Basidiospores (2.5 - 4.5 x 6 - 9 µm) are cylindric, hyaline, smooth, inamyloid and thin-walled

(Figure 1D). These mentioned morphological details agreed with the characters of *Lentinus squarrosulus described in* [10-13]. In addition, the sequence of 685 bp ITS region of the fungus showed 99 % identity with the *Lentinus squarrosulus* strain WCR1201. It is an edible mushroom commonly found in the wild and has recently draw attention as its potential use in nutraceutical products [18]. Its fruiting body contains high amount of proteins, sugars, lipid, amino acids, vitamin B, C, and D, and minerals [19]. Its mycelial biomass produced by submerged fermentation can be used as a source of bioactive compounds such as antioxidant activities and antiulcer potential [20]. However, it has not been cultivated on large scale for production of fruiting bodies [20].

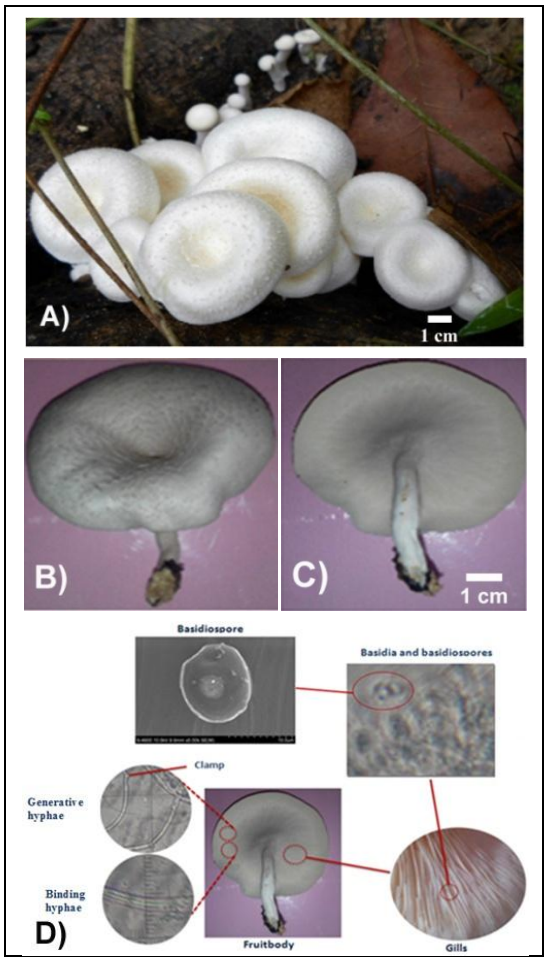

Figure 1. Fruiting body of the tough mushroom in the wild (A), pileus surface (B) and gill (C), and micro-morphological characters of basidiospore, hyphae and clamp (D).

3.2. Effects of different grains on spawn production

The mycelial growth of *L. squarrosulus* on the three different cereal grains was presented in Figure 2. Data were presented in mean (± SE) of the linear mycelial growth (mm/day). On the grain subtracts supplemented with $CaCO_3$ (1 %) and $MgSO_4$, (1 %), the mushroom mycelia grew significantly faster than the mycelia on the grain substrates without the supplementation ($p \leq 0.05$). There was no significantly difference in the mycelia growth rates on the unhusked rice,

brown rice and corn grain (8.26 ± 0.17, 8.06 ± 0.26, 8.07 ± 0.19, respectively, at $p \geq 0.05$) (Figure 2). However, there was a markable difference in the mycelial density on the unhusked rice giving the thickest white mycelia coated on the substrate surface, followed by the brown rice, and then the corn grain (Figure 3). In the study, the mycelial growth rate of the mushroom was found to be significantly slow (28.8 mm) at 20 ± 2 °C while it was recorded to be 57.6 mm at 30 ± 2°C after 7 days of culture on the PGA plates. Isikhuemhen et al. [18] and Adesina et al. [21] also reported that *L. squarrosulus* prefers to grow best vegetatively at temperature of 30 ± 2 °C and is one of a high temperature tolerant white rot fungus.

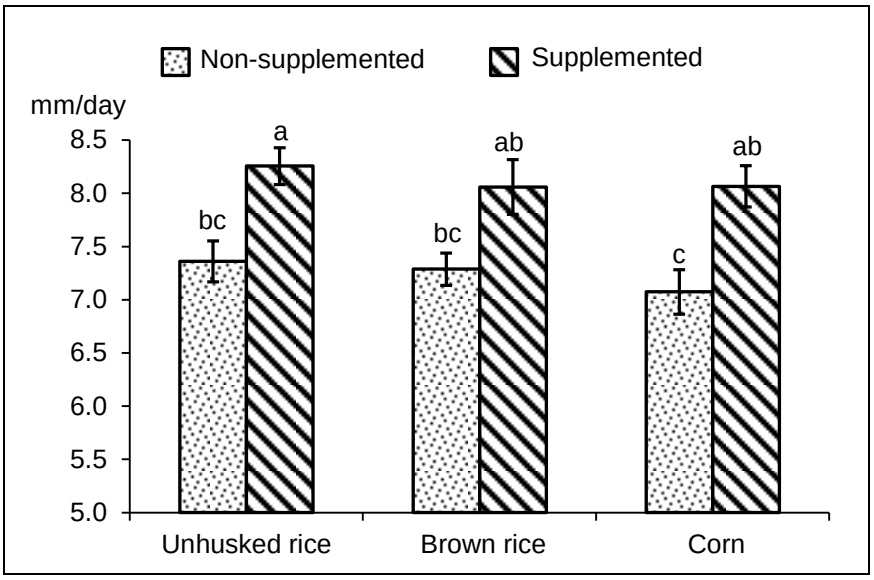

Figure 2. Effect of different grains on linear mycelial growth of *L. squarrosulus* on unhusked rice, brown rice and corn non-supplemented compared to those supplemented with $CaCO_3$ (1 %) and $MgSO_4$ (1 %). *Means* of the growth ± SE *(mm/day) followed* by *same letter* do not significantly differ (P = 0.05, LSD).

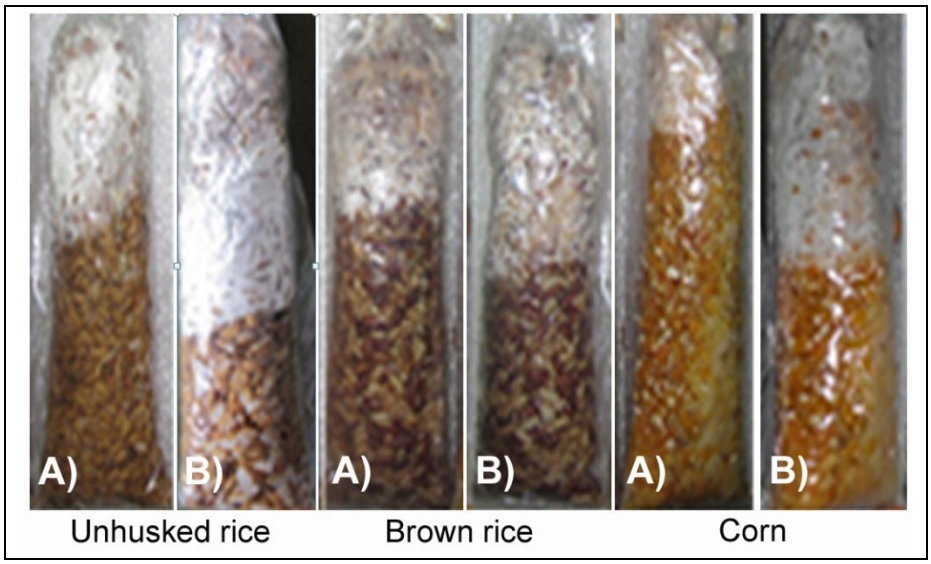

Figure 3. The linear mycelial growth of *L. squarrosulus* on unhusked rice, brown rice and Corn non-supplemented (A) and supplemented (B) with $CaCO_3$ (1 %) and $MgSO_4$ (1 %).

3.3. Effects of substrates on the growth and yield of fruiting bodies

Mycelial growth and fruiting body formation of *L. squarrosulus* on the three different commonly available substrates (sugar bagasse, rice straw and rubber tree sawdust) were presented in Table 1. On the sugar bagasse and rice straw substrates, the growth rate and full colonization of the mycelia on both of the substrates (8.4 mm/day and 24 days, respectively) were significantly faster than that on the sawdust substrate (7.4 mm/day and 27 days, respectively). However, the mycelial density and fresh fruiting body yield on the sawdust (331.6 g/bag) were significantly higher than that on both of sugar bagasse and rice straw (202.4 - 226.3 g/bag) (Table 1). Therefore, the sawdust material supplememted with corn bran and earthworm dung were conducted to investigate the mycelia growth and fruiting body production (Table 1). In the experiment, mycelial growth rate was enhanced (7.9 - 8.4 mm/day) when the supplements of corn bran (5 - 10 %) and earthworm dung (5 %) were used. Higher concentrations of the suppelments made the mycelial growth rate slower and full colonization in the material bags lasted longer than 24 days of incubation. The maximun yields of the fresh fruiting bodies archived on the sawdust media supplemented with corn bran (10 %) and earthworm dung (5 %) were 411.5 and 397.6 g/bag, respectively (Figure 4).

Table 1. Substrate and supplement effects on mycelial growth rate and fruiting body production of *L. squarrosulus.*

Substrates	Mycelial growth rate (mm/day)*	Full mycelial colonization (day)*	Fresh weight of fruiting bodies (g/kg substrate)*
Sugar bagasse	$8.42^{a} \pm 0.07$	$23.84^{a} \pm 0.21$	$226.30^{e} \pm 4.56$
Rice straw	$8.39^{a} \pm 0.09$	$24.00^{a} \pm 0.26$	$202.36^{d} \pm 4.54$
Rubber tree sawdust	$7.42^{c} \pm 0.09$	$27.02^{c} \pm 0.34$	$331.58^{c} \pm 5.14$
Sawdust + corn bran (5 %)	$7.94^{b} \pm 0.08$	$25.12^{b} \pm 0.28$	$358.57^{b} \pm 5.37$
Sawdust + corn bran (10 %)	$8.37^{a} \pm 0.07$	$23.66^{a} \pm 0.18$	$411.48^{a} \pm 5.28$
Sawdust + corn bran (20 %)	$3.94^{e} \pm 0.08$	$44.29^{e} \pm 0.77$	$395.45^{a} \pm 5.78$
Sawdust + earthworm dung (5 %)	$8.35^{a} \pm 0.08$	$24.26^{a} \pm 0.28$	$397.61^{a} \pm 5.16$
Sawdust + earthworm dung (10 %)	$6.01^{d} \pm 0.07$	$33.63^{d} \pm 0.41$	$369.09^{b} \pm 5.28$

**Means ± SE followed by the same letter in a column are not significantly different at $P \geq 0.05$.*

L. squarrosulus, one of a high temperature tolerant white rot fungus found in many parts of Asia (Malaysia, Philippine, India and Viet Nam), Oceania (Papua New Guinea) and across sub-Saharan Africa (Nigeria), is abundant growing on decaying wood in the tropical rain forest [10-13, 17, 18, 21]. Rubber wood *waste,* one of the *most* abundant *lignocellulosic* materials is always available for mushroom cultivation in Viet Nam. Therefore, wood waste material such as rubber tree sawdust is more suitable for the fungus cultivation than rice straw, banana leaves, coconut leaves, coconut coir dust, and bagasse materials [22]. It is also reported that the fungus can grow on waste logs, stem barks and leaves of several fruit trees such as *Spondias mombi, Cirus sinensis, Terminalia cattapa, Mangifera indica, Anacardium occidentalis* and *Psdium guajava* [21]. In addition, besides corn bran and earthworm dung used as supplements in the study, several other suitable supplements for the growth of this mushroom such as rice bran, oil palm

waste fiber, fresh cassava flour, poultry droppings, cow dung and horse dung were also reported [21]. Moreover, *L. squarrosulus* is recently attracting attention due to its rapid mycelia growth and potential for use in food, nutraceutical products and biodegradation in many countries across sub-Saharan Africa and many parts of Asia [18].

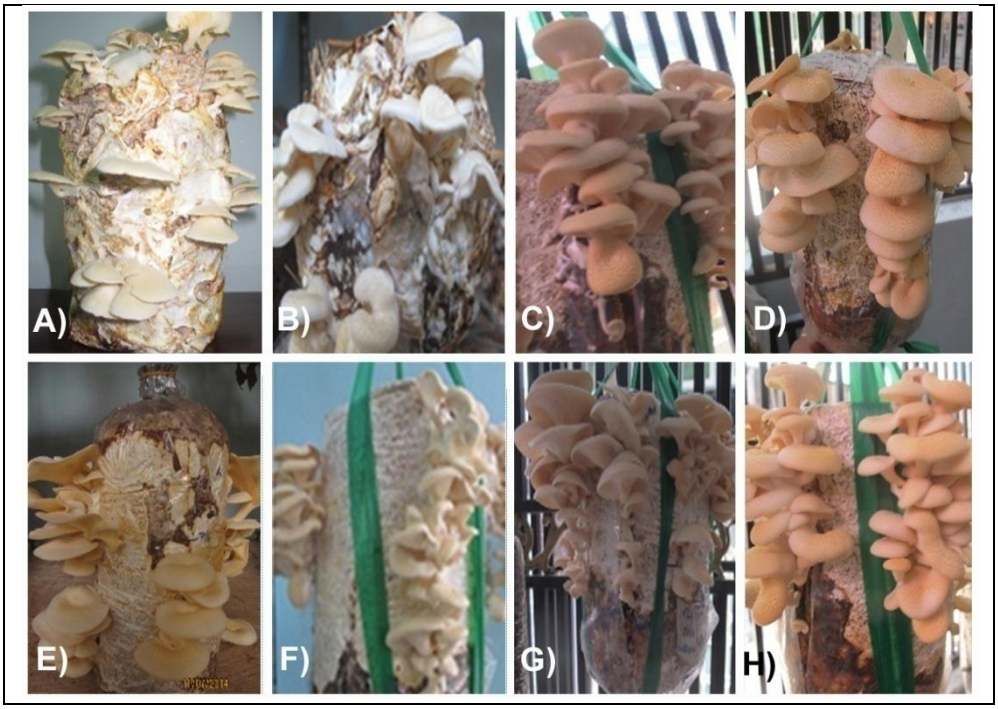

Figure 4. Fruiting body formations of *L. squarrosulus* on different substrates, sugar bagasse (A), rice straw (B), sawdust (C), sawdust + 5 % corn bran (D), sawdust + 10 % corn bran (E), sawdust + 20 % corn bran (F), sawdust + 5 % earthworm dung (G) and sawdust + 10 % earthworm dung (H).

3.4. Nutritional valuation of *L. squarrosulus*

The wild edible mushroom, *L. squarrosulus*, was found to abundantly grow in the rainy season in Tan Bien district, Tay Ninh province. Due to its vigorous growth and delicious taste, the nutritional value of the mushroom was determined. Total protein, fat, carbohydrate and ash of the mushroom were reported as the percentage compositions per 100 g of the dry fruiting bodies (Table 2).

Table 2. Nutritional compositions (%)* of *L. squarrosulus*.

Species	Protein	Crude fat	Carbohydrates	Crude total ash
L. squarrosulus	30.09 ± 0.94	5.83 ± 0.28	40.78 ± 1.62	6.23 ± 0.36

*Percentage ± SE (per 100 g of the dry fruiting bodies)

The nutritional analysis of *L. squarrosulus* showed that carbohydrate and protein contents were the most abundant nutrients, 40.78 and 30.09 %, respectively, while crude fat and total ash were the least abundant nutrients, 5.83 and 6.23 %, respectively. This strain has lower

carbohydrate, but higher protein and fat contents than those of the strain from Ghana (80.17, 19.43 and 1.98 %, respectively) [23]. Moreover, it was reported that the protein content of *L. squarrosulus* is remarkbly higher than that of other commercially cultivated species such as *Auricularia* sp. (4.2 %), *Lentinus laedodes* (13.4 - 17.5 %), *Pleurotus florida* (27.0 %) and *Volvariella volvacea* (25.9 %) [4]. The *L. squarrosulus* carbohydrate content (40.78 %) is lower than that of some common edible mushrooms such as *Auricularia* sp. (82.8 %), *Lentinus edodes* (67.5 - 78.0 %), *Pleurotus florida* (58 %) and *Volvariella volvacea* (45.3 %) [4]. However, its lipid content (5.83 %) is relatively low compared with *Agaricus bisporus* (8%), *Auricularia sp.*(8.3%), *Lentinus edodes* (4.9 - 8.0 %) [4, 11]. The *L. squarrosulus* is wide distributed in the wild in all 3 regions, the *North, Central and South of Viet Nam. It* has been collected and used as food for years by the local people in our country, in several other parts of Asia such as Malaysia, Philippine and India, in Oceania and across sub-Saharan Africa [10-12, 17, 20-22]. The mycelial extract of *L. squarrosulus* was reported to have good antioxidant properties *in vitro* [24] and no toxic effects, even at high doses (5 g/kg) to Sprague Dawley rats [20, 25]. Although the species has been catalogued in the list of edible mushrooms by Trinh Tam Kiet [13], the semi-chronic toxicity of the mushroom extract needs to be studied for getting a better understanding of the long-term effects of the wild tough mushroom.

4. CONCLUSION

The wild tough mushroom strain collected in Tan Bien, a rural district of Tay Ninh Province in the southeast of Viet Nam is *Lentinus squarrosulus*, a tropical white rot fungus belonging to family Polyporaceae, order Polyporales, class Agaricomycetes, Phylum Basidiomycota. This study show that the unhusked rice supplemented with $CaCO_3$ (1 %) and $MgSO_4$ (1 %) was the most appropriate formula for mycelial growth of the mushroom. *L. squarrosulus* can be successfully cultivated in the laboratory on the rubber tree sawdust for the mycelial growth and fruiting body formation. The spawn production and fruiting bodies of *L. squarrosulus* can also be enhanced by using agricultural waste like corn bran and earthworm dung as supplements.

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